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Growth factor dependent cholinergic function and survival in primary mouse spinal cord cultures ☆

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This article is dedicated to the memory of Dr. Robert E. Sheridan

Abstract

In primary embryonic spinal cord cultures, synaptic transmission can be conveniently studied by monitoring radiolabeled neurotransmitter release or by recording of electrophysiological responses. However, while the mature spinal cord contains an appreciable number of cholinergic motoneurons, cultures of embryonic spinal cord have a paucity of these neurons and release little or no acetylcholine upon stimulation. To determine whether the proportion of cholinergic neurons in primary mouse spinal cord cultures can be augmented, the effects of several classes of growth factors were examined on depolarization- and Ca^{2+}-evoked release of choline/acetylcholine (Ch/ACh). In the absence of growth factors, little or no evoked release of radiolabeled Ch/ACh could be demonstrated.

Media supplemented with brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) or basic fibroblast growth factor (bFGF) were examined for their ability to preserve the population of neurons in culture. CNTF was found to increase the number of surviving neurons and to enhance the release of radiolabeled Ch/ACh; the other factors were without effect. The action of CNTF was transient, and the neuronal population decreased to levels observed in cultures lacking growth factor after 20 days in vitro. The correlation between enhanced neuron survival and increased Ch/ACh release suggests that CNTF protected cholinergic neurons, albeit transiently, from cell death.
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Keywords: Mouse; Spinal cord; Cell culture; Growth factor; CNTF; BDNF; bFGF; Choline; Acetylcholine; Glycine

Introduction

Primary cultures of dissociated embryonic mammalian spinal cord represent a convenient system to study mechanisms of transmitter release. Radiolabeled neurotransmitters or precursors are internalized by neurons in culture and released quantitatively in response to depolarization and Ca^{2+}; this release is inhibited by tetanus toxin and botulinum neurotoxins in a concentration- and time-dependent manner (Bergey et al., 1987; Williamson et al., 1992; Keller et al., 2004; Hall et al., 2004; Sheridan et al., 2005). The sensitivity of spinal cord cultures to botulinum neurotoxin (BoNT) appears to be greater than that of other cell systems described, and spinal cord motoneuron terminals represent an important biological target for BoNT (Daniels-Holgate and Dolly, 1996; Simpson, 2004). Using these cultures, it has been possible to demonstrate a relationship between toxin-mediated cleavage of SNARE proteins and inhibition of transmitter release (Williamson et al., 1996; Keller et al., 1999, 2004), even though the cultures contain a large proportion of non-neuronal cells which lack regulated exocytosis (Brenneman et al., 1987). The majority of transmitter release studies have focused on depolarization-

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dependent release of glutamate or glycine, neither of which is the normal target of BoNT in vivo (Simpson, 2004). Unless spinal cord neurons are cocultured with muscle or cervical ganglion cells, little or no cholinergic activity has been observed (Bennett et al., 1980; Kobayashi et al., 1987; Wang et al., 1990; Braun et al., 1997).

Spinal cord preparations usually include dorsal root ganglion (DRG) cells, which are important in stabilizing synapses between neurons and skeletal muscle (Kobayashi et al., 1987); however, DRG cells alone are not capable of maintaining the population of cholinergic neurons (Wang et al., 1990). During the first weeks after plating, large numbers of neurons undergo cell death, the majority of which appear to be cholinergic motoneurons (Ransom et al., 1977). It has been reported that brain derived neurotrophic factor (BDNF) can enhance the short term (2–5 days) survival of spinal cord motoneurons in vitro (Wong et al., 1993; Henderson et al., 1993; Hughes et al., 1993). Similarly, basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) have been reported to support the population of motoneurons in culture (Hughes et al., 1993; Wong et al., 1993), although the results have been inconsistent.

The current study is an attempt to determine the ability of neurotrophic factors to promote the survival of cholinergic neurons in primary mouse embryonic spinal cord cultures. CNTF was found to increase the number of surviving neurons and to enhance the release of radiolabeled choline/acetylcholine (Ch/ACh), while the other factors were without effect. The action of CNTF was transient, being significant only during week 2 in culture. These results suggest that CNTF provides a limited window for studies on cholinergic neurons in primary mouse spinal cord cultures.

Materials and methods

Spinal cord cultures

Spinal cord with DRG were removed from embryonic Hsd: NIHS Swiss mice at gestation day E13–E14, dissociated in 0.125% trypsin and plated at a density of 4×10^5 cells/cm^2 in 12-well collagen-coated tissue culture plates. The cells were cultured at 37 °C in 90% air/10% CO_2 in Eagle's Minimum Essential Media (MEM) with 5% horse serum and N3 supplement (Romijn et al., 1981; Wang et al., 1990) or in neurobasal media with B27 supplement and 2% horse serum (Brewer, 1995). Cell cultures were treated with 54 μM 5-fluoro-2′-deoxyuridine and 140 μM uridine 5–9 days after plating to inhibit glial cell division. Thereafter, cells were fed twice per week. Growth factors were added to the culture media 2 days after plating and reapplied at each media change.

Determination of neuronal cell density

To identify the neuronal population in culture, the media was aspirated and cells were washed twice with phosphate-buffered saline, fixed in 4% paraformaldehyde for 30 min at room temperature and stained for neuron specific enolase (NSE) using the method of Brenneman et al. (1987). NSE positive cells were counted under a phase contrast microscope at 160×. Cells from 20 fields were averaged, and 4 wells were counted for each condition.

Neurotransmitter release assay

Neurotransmitter release was detected by the method of Williamson et al. (1992) and is described in detail by Sheridan et al. (2005). The growth medium was removed, and the cells were incubated at 37 °C for 20 min in a HEPES-buffered salt solution (HBSS) containing ^{3}[H]-glycine, ^{3}[H]-glutamate, ^{3}[H]-glutamine or ^{14}[C]-Ch, as appropriate. The composition of the HBSS was 135 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES/NaOH (pH 7.3) and 0.1% w/v bovine serum albumin. The cells were then washed briefly with HBSS and sequentially incubated for 5 min each in (1) Ca^{2+}-free HBSS containing 0.5 mM EGTA, (2) HBSS containing 56 mM K^+ + 2 mM Ca^{2+} and (3) Ca^{2+}-free HBSS containing 0.5 mM EGTA. Each incubation solution was collected and the radioactivity was determined by scintillation counting. Following these incubations, the cells were lysed in 0.2% sodium dodecyl sulfate and the remaining radiolabel in the cells was determined. The labeled neurotransmitter selectively released with high (56 mM) K^+ in the presence of 2 mM Ca^{2+} was expressed as a fraction of the total radioactivity in the cells. Cells incubated with ^{14}[C]-Ch release ACh as well as unmodified Ch (McGee et al., 1978). Since we did not separate these products, the materials released after incubation with ^{14}[C]-Ch are designated collectively as Ch/ACh in the present study.

Data analysis

Unless stated otherwise, all data are expressed as the mean ± SEM. Statistical analysis was performed using a one-way analysis of variance followed by Bonferroni's posttests (Graphpad InStat ver 3.0, San Diego, CA, USA). $P \leq 0.01$ was considered to be statistically significant.

Materials

MEM, Neurobasal media, B27, horse sera, 5-fluoro-2′-deoxyuridine and uridine were obtained from Invitrogen Corp. (Carlsbad, CA, USA). BDNF (human recombinant), CNTF (rat recombinant), and routine reagents were purchased from Sigma-Aldrich (St. Louis MO, USA). Bovine recombinant bFGF was obtained from Roche Applied Science (Indianapolis, IN, USA). Growth factors were applied at concentrations twice their EC50, as provided by the supplier. Radiolabeled neurotransmitters and precursors were obtained from Perkin Elmer Life and Analytical Sciences (Shelton, CT, USA).

Results

Cell death in spinal cord cultures

Following the initial plating of mouse embryonic spinal cord cells, there was a substantial loss of neurons before the

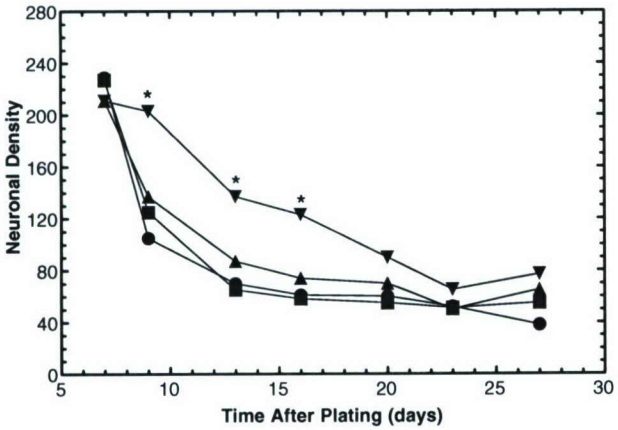

Fig. 1. Effect of growth factors on neuronal survival. Spinal cord neurons were counted in 20 random 160× microscopic fields at the indicated times after plating under control conditions (●) or following treatment with bFGF (■, 2 ng/ml), BDNF (▲, 1 ng/ml) or CNTF (▼, 10 ng/ml). Growth factors were added 2 days after plating. The symbols represent the mean number of cells per field obtained from 4 cultures. Asterisks indicate differences that are significantly different from control ($P \leq 0.01$).

population stabilized. From day 7–27 in vitro, the number of neurons decreased by 81% (Fig. 1). Treatment of cultures with CNTF (10 ng/ml) resulted in a marked increase in the number of surviving neurons relative to control. This increase was statistically significant at 9, 13 and 16 days after plating ($P \leq 0.01$). Treatment of cells with BDNF (1 ng/ml) or bFGF (2 ng/ml) failed to produce significant changes in the survival of cells above those observed under control conditions. However, even in CNTF, viable neurons decreased during culture, and there was no significant difference in the number of neurons relative to control at times ≥20 days. Cells surviving through the first month were viable for the next three months in culture, as noted by previous investigators (Ransom et al., 1977; Keller et al., 1999). This pattern of cell survival was essentially the same in either MEM or Neurobasal media.

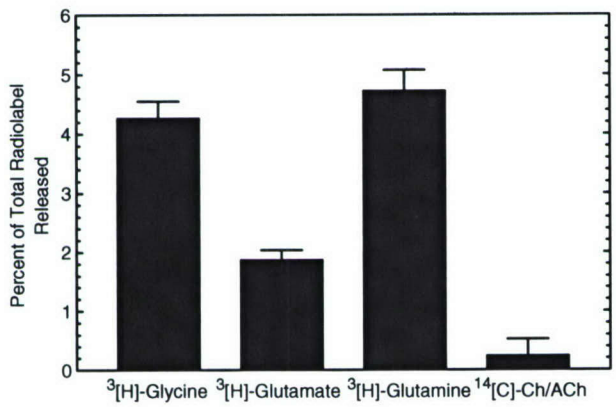

Fig. 2. Evoked release of neurotransmitters. Spinal cord cultures at 9 days post-plating were washed in HBSS, incubated with the appropriate radiolabeled neurotransmitters or precursors and stimulated in 56 mM K^+ in the presence of 2 mM Ca^{2+}. Cells were grown in MEM supplemented with N3 and 5% horse serum without additional growth factors. Bars represent the mean ± SEM of 6 experiments.

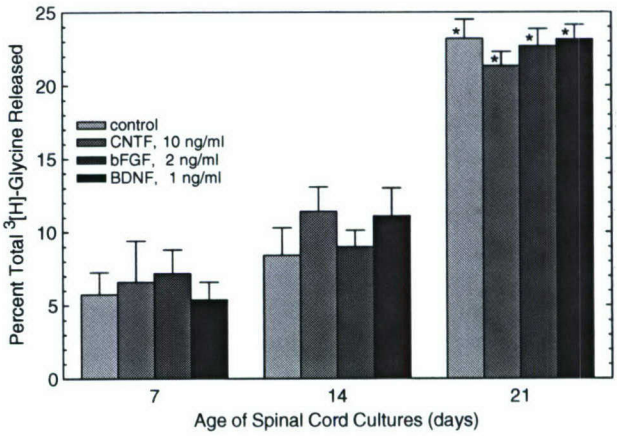

Fig. 3. Time-dependent increase in glycine release. Evoked release of ^{3}[H]-glycine from cells treated with CNTF, bFGF or BDNF was measured at 7, 14 or 21 days after plating. There was a pronounced increase in the amount of ^{3}[H]-glycine released over this time period, especially between 14 and 21 days, although no consistent effect of the growth factors was observed. The cells were cultured in Neurobasal media supplemented with B27 and 2% horse serum. Bars represent the mean ± SEM of 5 experiments. Asterisks indicate differences that are significantly different from ^{3}[H]-glycine released at 7 days ($P \leq 0.01$).

Depolarization- and Ca^{2+}-dependent neurotransmitter release

Cultures grown in MEM with N3 supplement at 9 days in vitro are capable of taking up and releasing approximately 5% of total incorporated glycine as indicated in Fig. 2. Uptake of either ^{3}[H]-glutamate or ^{3}[H]-glutamine was also observed and led to evoked release of 2–5% of the total incorporated label. However, although ^{14}[C]-Ch was taken up by these cultures, evoked release of labeled Ch/ACh was barely detectable (Fig. 2).

As cultures matured over a 3-week period, there was an increase in the percentage of ^{3}[H]-glycine released by K^+-induced

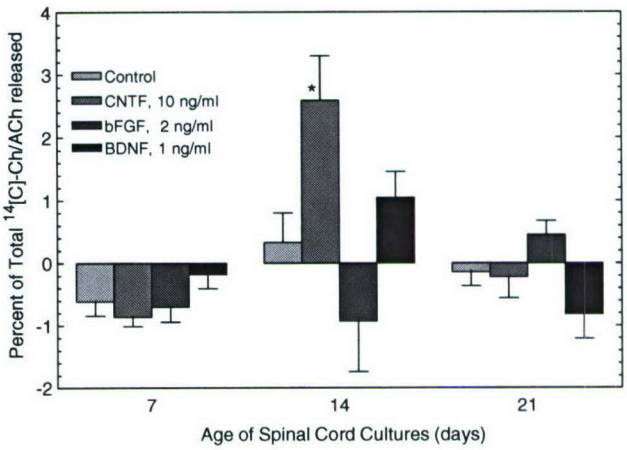

Fig. 4. Time-dependent Ch/ACh release. Evoked release of Ch/ACh from cells treated with CNTF, bFGF and BDNF was measured at 7, 14 and 21 days after plating. Although there was an increase in the amount of Ch/ACh released in cultures treated with CNTF at 14 days, this effect was transient and no significant Ch/ACh release could be elicited at times ≥21 days. Cells were grown in MEM supplemented with N3 and 5% horse serum, but similar results were also observed with cells cultured in Neurobasal medium with B27 and 2% horse serum. Bars represent the mean ± SEM of 5 experiments. Asterisk indicates significant difference from value at 7 days in culture ($P \leq 0.01$).

depolarization in the presence of normal extracellular Ca^{2+}. As illustrated in Fig. 3, this increase occurred predominantly between 14 and 21 days in culture and was not affected significantly by the presence of CNTF, bFGF or BDNF. Paradoxically, enhanced ^{3}[H]-glycine release occurred during the time when the total number of neurons in the cultures underwent marked reductions. Decrements in release expected from the loss of neurons may have been compensated by a continued expansion of dendritic processes from surviving cells (Ransom et al., 1977; Williamson et al., 1992). In addition, it is possible that fewer glycinergic neurons were lost during the time of rapid cell death, resulting in a higher percentage of glycinergic cells in the total population at day 21.

CNTF transiently increases Ch/ACh release

In contrast to the results obtained with ^{3}[H]-glycine release, evoked release of labeled Ch/ACh was generally undetectable in the absence of added CNTF (Fig. 4). This was found in both MEM and Neurobasal culture media and at all times up to 5 weeks after isolation of cells. However, even in CNTF, release of Ch/ACh was consistently observed only at two weeks in vitro. At this time, there were significant differences in the number of surviving neurons between control and CNTF-treated cultures, although the maximum difference occurred at day 7 (Fig. 1). Combinations of CNTF with BDNF or bFGF did not improve Ch/ACh release compared with treatment with CNTF alone (data not shown).

To establish that the 10 ng/ml CNTF used in this study was optimal, cultures were grown in the presence of 0.01 to 100 ng/ml CNTF for 12 days, starting on culture day 2, and assayed for high K^{+}-evoked Ch/ACh release. Fig. 5 shows the effect of CNTF concentrations on release of radiolabeled Ch/ACh at the 2-week time window of optimum cholinergic response. There was a significant increase in Ch/ACh release in the presence of CNTF concentrations >0.1 ng/ml, with maximal enhancement of release occurring at 10 ng/ml.

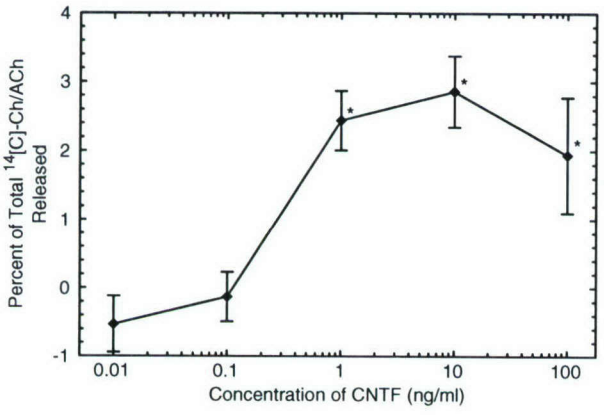

Fig. 5. Effect of CNTF on Ch/ACh (♦) release. Cells were cultured in Neurobasal media supplemented with B27 and 2% horse serum. Cultures were treated with CNTF at the indicated concentrations starting on day 2. At 14 days in vitro, cells were loaded with ^{14}[C]-Ch, and evoked release in the presence of 54 mM K^{+} and 2 mM Ca^{2+} was determined as a percent of total labeled transmitter. CNTF increased ^{14}[C]-Ch/ACh release in a concentration-dependent manner with 10 ng/ml being optimal. Symbols represent the mean ± SEM of data from 6 experiments. Asterisks indicate differences that are significantly different from control values ($P \leq 0.01$).

Discussion

There was a pronounced time-dependent decline in the number of neurons surviving in primary mouse spinal cord cultures, especially during the first two weeks after plating (Fig. 1), in agreement with previous investigators (Ransom et al., 1977; Bennett et al., 1980; Comella et al., 1994). Although the number of viable neurons in the cultures decreased, evoked release of neurotransmitters, notably glycine, increased during this time as indicated in Fig. 3. The increase in transmitter release parallels enhancements in neurite density, metabolic activity and electrical excitability (Ransom et al., 1977; Williamson et al., 1992). It is likely that the ability of the cultures to release neurotransmitters is dependent upon the development of an extensive field of neurites and synaptic connections. For release of glycine or glutamate, these factors more than compensate for the loss of neurons between initial isolation and development of a more mature culture. However, the selective loss of cholinergic neurons makes it difficult to elicit Ch/ACh release from spinal cord cultures in the absence of stabilization by coculture with target cells (Bennett et al., 1980; Kobayashi et al., 1987; Wang et al., 1990; Braun et al., 1997).

The presence of CNTF transiently delayed the death of viable neurons, with the maximum enhancement of cell survival occurring 9–16 days in vitro (Fig. 1). This enhanced survival was not detected in the presence of other neurotrophic factors such as BDNF or bFGF. Evoked release of Ch/ACh could be observed in the spinal cord cultures within a narrow window at 2 weeks in vitro (Fig. 4), in the presence of CNTF concentrations >0.1 ng/ml (Fig. 5). A plausible interpretation is that this restricted window represents the time between expansion of neurites and synaptic contacts, which increases overall evoked transmitter release, and the progressive deletion of cholinergic neurons, which results in the loss of Ch/ACh release. Although CNTF helped to stabilize the population of neurons up to day 16 in vitro, it could not prevent cell death at later time points. The reduction of Ch/ACh release in CNTF-treated cultures at ≥3 weeks is consistent with previous reports that cholinergic neurons represent a major population lost in cultures of embryonic spinal cord cells (Comella et al., 1994).

The finding that CNTF was effective in preserving evoked release of Ch/ACh, even transiently, was unexpected. Although CNTF has been implicated in maintaining motoneuron development in vivo (Forger et al., 1993; Masu et al., 1993), its efficacy in cultured cells has not previously been demonstrated (Hughes et al., 1993; Wong et al., 1993; Zurn et al., 1996). Of the growth factors examined, BDNF has been associated most often with enhanced motoneuron survival in vitro (Henderson et al., 1993; Hughes et al., 1993; Wong et al., 1993; Zurn et al., 1996; Kwon and Gurney, 1996). It should be noted that most of these in vitro studies examined periods only up to 5 days in culture. This would be too brief to show appreciable evoked neurotransmitter release in the assay employed in the current study.

Although CNTF was found to be beneficial in preserving cholinergic function, the brief window of cholinergic neurotransmission would limit the nature of studies that could be undertaken. A more stable response would be desirable to study problems such as BoNT persistence in cholinergic motoneuron terminals that represent their primary in vivo target. Perhaps a combination of treatment by CNTF and coculture with potential targets such as skeletal muscle may lead to the required stability of cholinergic responses.

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